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Review

Beyond ribosome rescue: tmRNA and co-translational processes

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ABSTRACT

tmRNA is a unique bi-functional RNA that acts as both a tRNA and an mRNA to enter stalled ribosomes and direct the addition of a peptide tag to the C terminus of nascent polypeptides. Despite a reasonably clear understanding of tmRNA activity, the reason for its absolute conservation throughout the eubacteria is unknown. Although tmRNA plays many physiological roles in different bacterial systems, recent studies suggest a general role for *trans*-translation in monitoring protein folding and perhaps other co-translational processes. This review will focus on these new hypotheses and the data that support them.

tmRNA is a specialized tRNA-like molecule that has the unique ability to mediate the addition of a peptide tag to the C terminus of nascent polypeptides before they are released from the ribosome. In a reaction known as *trans*-translation, tmRNA enters substrate ribosomes and acts both as a tRNA and an mRNA, accepting the nascent polypeptide, and encoding the peptide tag that is added by the ribosome. tmRNA and other factors required for *trans*-translation are conserved throughout the bacterial kingdom, indicating that the reaction provides a significant competitive advantage. Despite a reasonably clear understanding of the activity of tmRNA, the reason this reaction has been conserved throughout bacterial evolution is unknown. Although tmRNA plays many physiological roles in different bacterial systems, recent studies on tmRNA substrate selectivity and on how ribosomes behave in the absence of tmRNA have suggested a general role for *trans*-translation in monitoring protein folding and perhaps other co-translational processes. This review will focus on these new hypotheses and the data that support them.

The 5′ and 3′ ends of tmRNA fold into a structure that mimics the acceptor and WC arms of alanyl-tRNA [1,2]. The 5′ and 3′ termini are processed by RNase P, RNase E, and exoribonucleases in the same manner as tRNAs [1,3,4]. Consistent with this $tRNA^{Ala}$-like structure, tmRNA is charged with alanine by AlaRS in vitro and in vivo, and is bound by EF-Tu [1,5,6]. However, tmRNA is much larger than a tRNA (363 nt in *Escherichia coli*), and does not have a D arm or anticodon arm. Instead, tmRNA contains 3–4 pseudoknots and a specialized open reading frame encoding the peptide tag [1,7–9]. In addition to EF-Tu, tmRNA is bound by a small protein, SmpB [10]. SmpB has structures that mimic the anticodon arm of a tRNA, and is required for tmRNA activity [10–12]. During *trans*-translation, alanyl-tmRNA bound to SmpB and EF-Tu enters the A-site of a substrate ribosome (Fig. 1). What makes a ribosome a substrate for tmRNA is discussed below, but because initiation of translation on tmRNA has not been observed, substrate ribosomes are assumed to have a peptidyl-tRNA in the P-site. Cryo-EM and chemical probing experiments suggest that during accommodation in the A site, the tRNA-like domain of tmRNA is located near the peptidyl-transfer active site and SmpB is near the decoding center [13–16]. The nascent polypeptide is transpeptidated to tmRNA, and the tag reading frame is inserted in the decoding center of the ribosome. Translation resumes at an alanine codon at the 5′ end of the tag reading frame, and continues to a stop codon, releasing the tagged protein product. Because the tag reading frame is in the middle of tmRNA, translation of this sequence after the tRNA-like domain has passed through the ribosome presents a topological challenge. It is not known whether the entire tmRNA sequence enters the ribosome, or whether local unfolding allows some of the molecule to remain outside. In either case, it appears likely that structural changes in the ribosome are necessary both to orient the open reading frame in the mRNA channel and to allow translation to continue through the tag reading frame. The net result of *trans*-translation is removal of the substrate translational complex. The ribosomal subunits are dissociated and released,

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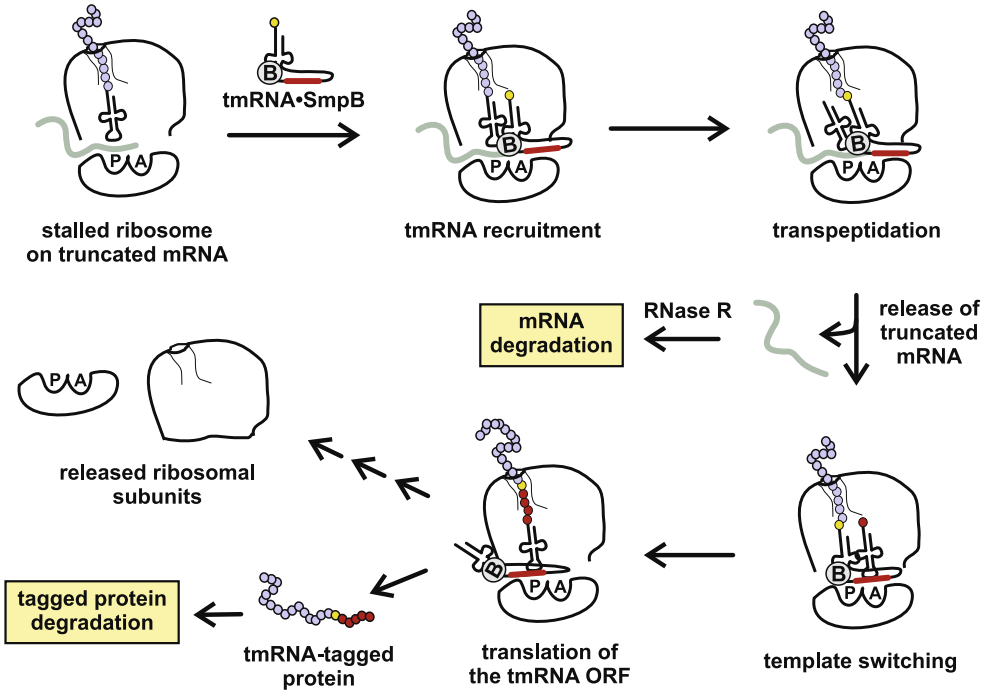

Fig. 1. The *trans*-translation model of tmRNA activity. The tmRNA·SmpB complex is recruited with EF-Tu (not shown) to the A-site of substrate ribosomes. Acting like a tRNA, tmRNA accepts the nascent polypeptide in a normal transpeptidation reaction. The tmRNA open reading frame (ORF) replaces the mRNA in the decoding center, and the mRNA is released and degraded. Translation resumes using the tmRNA ORF as a template. After synthesis of the tmRNA-encoded peptide, the tagged protein is released for degradation by cellular proteases, and ribosomal subunits are recycled for further rounds of translation.

the mRNA is released from the ribosome and degraded, and the tagged protein is targeted for proteolysis by protease recognition sites in the tag peptide [17–23].

tmRNA and SmpB homologs have been identified in every sequenced bacterial genome, including those with severely reduced genome sizes [24,25]. This conservation suggests that *trans*-translation evolved early in the bacterial lineage, and provides a significant competitive advantage in every environment that supports bacterial life. However, eukaryotes and archaea do not have tmRNA or SmpB (except in some organelles). The *trans*-translation reaction also occurs with high frequency in growing cells. Estimates from *E. coli* suggest that 0.4% of translation reactions are terminated by *trans*-translation [26]. What, then, is the critical function of *trans*-translation in bacteria? Mutations that inactivate tmRNA or SmpB are lethal in some species, including *Neisseria gonnorhoeae* and *Shigella flexneri* [27] (K.C. Keiler, unpublished results). In other species, phenotypes include deficiencies in virulence, sporulation, cell cycle progression, antibiotic resistance, and stress responses [28]. How can this wide array of effects be explained by removal of a single ubiquitous process? Explanations for individual phenotypes have centered on two hypotheses: tmRNA is required to rescue stalled ribosomes and maintain the translational capacity of the cell, and tmRNA is required for correct regulation of genetic circuits. The ribosome rescue hypothesis provides an explanation for why tmRNA is so broadly conserved, but recent data indicate that the simplest models for producing phenotypes with stalled ribosomes are not correct. Molecular data from several systems, described below, indicate that some phenotypes are due to misregulation of specific regulatory proteins in the absence of tmRNA. However, it is not yet clear how general these mechanisms are or how many phenotypes may be caused by problems with individual genetic circuits. Exciting new data suggest a third possibility: *trans*-translation may be coordinated with other crucial co-translational processes, such as protein folding and secretion. The bases of these hypotheses and the data that support or refute them are entwined with the question of how substrates for *trans*-translation are generated.

In vitro studies indicate that tmRNA can efficiently enter translating ribosomes if they have reached the 3′ end of the mRNA [29]. On ribosomes stalled after translating a 5 residue peptide in vitro, tmRNA activity was most efficient when there was <6 nt of mRNA extending 3′ of the P-site. When the mRNA extended >15 nt 3′ of the P-site, essentially no activity was observed, suggesting that tmRNA activity is inhibited by mRNA extending past the leading edge of the ribosome. These observations have not yet been confirmed using ribosomes with larger nascent polypeptides, but they are consistent with most of the known tmRNA substrates in vivo. A wide variety of proteins have been intentionally targeted to tmRNA by inserting a strong transcriptional terminator before the stop codon. Expression of these genes produces a 'non-stop' mRNA in which the reading frame continues to the 3′ end, and tagging occurs after translation of the encoded protein [20]. There is even one naturally occurring example of such a substrate. Some environmental isolates of *Bacillus subtilis* have a mutation in the *kinA* gene that replaces the normal stop codon with a sense codon [30]. Because there is no additional in-frame stop codon before the intrinsic transcriptional terminator, a non-stop mRNA is likely to be produced. Strains with the non-stop *kinA* gene do not accumulate KinA protein under conditions where it is normally produced, consistent with tagging and proteolysis. In support of this idea, when *ssrA* (the gene encoding tmRNA) is deleted in these strains, KinA protein accumulates.

Other tmRNA substrates are likely to result from termination of transcription before the stop codon is reached. For example, the transcription factor LacI binds to an operator site within its own gene and blocks transcription elongation, leading to non-stop mRNAs [31]. Likewise, transcription elongation of *treP* in *B. subtilis* is blocked by binding of the transcription factor CcpA within the *treP* open reading frame, resulting in a non-stop mRNA and production of tagged TreP protein [32]. Non-stop messages are also

produced through activation of the so-called 'mRNA interferases', which are encoded by a subset of prokaryotic toxin-antitoxin modules. Some of these mRNA interferases, such as RelE, enter the A-site of the ribosome and promote cleavage of the mRNA [33]. Other toxins are sequence-specific RNases that preferentially cleave within single-stranded regions of mRNAs [34,35]. These toxin proteins are activated under stress conditions, including starvation, when resources need to be temporarily diverted from translation to other cellular activities. tmRNA activity is required for recovery from toxin-mediated stasis, likely because *trans*-translation efficiently removes the stalled ribosomes, nascent polypeptides, and mRNAs [36,37]. Additionally, degradation of tagged proteins to free amino acids may facilitate recovery from acute starvation stress.

Stalling during translation of intact messages can also lead to *trans*-translation. Depletion of a tRNA or release factor promotes tagging at the cognate sense and stop codons, and proteins that naturally contain rare codons or inefficient termination sequences are tagged at a high rate [38–45]. In these instances, translation complexes are converted into tmRNA substrates by RNases that truncate the transcript either within the A-site codon, or at the leading edge of the paused ribosome. A-site mRNA cleavage during translational stalling is not mediated by any of the known mRNA interferases, and has recently been shown to be dependent upon RNase II [46]. RNase II is the major 3′–5′ exoribonuclease responsible for mRNA degradation in *E. coli*. Though required for A-site cleavage, RNase II is unable to directly degrade mRNA into the ribosomal A-site. Instead, RNase II degrades mRNA from the 3′ terminus until it encounters the paused ribosome, producing a transcript that is truncated ~18 nt downstream of the A-site codon. Presumably, this mRNA degradation to the ribosome's leading edge facilitates subsequent cleavage of the A-site codon by an unidentified RNase. Although A-site cleavage is thought to provide a mechanism for tmRNA recruitment to paused ribosomes, tagging is unaffected by deletion of the *rnb* gene, which encodes RNase II. Translational pausing in RNase II^{-} cells results in mRNA cleavage at a position 12 nt downstream of the A-site, which is predicted to support tagging, albeit at a significantly reduced rate [29,46]. These studies suggest A-site mRNA cleavage may not necessarily be functionally linked to *trans*-translation. Indeed, A-site truncated transcripts typically only accumulate to high levels in *ssrA* cells, and it is possible that this unique mRNA processing plays a role in tmRNA-independent ribosome recycling. Other stalled ribosomes are not targeted to tmRNA. In particular, ribosomes stalled during attenuation or at programmed pausing sequences do not result in tagging, even though the mRNA downstream of the stalled ribosome is exposed to the same nuclease activities as substrate ribosomes. In at least two instances, the arrested ribosome complex includes a protein or tRNA in the A-site, which prevents entry of tmRNA. Ribosomes paused during translation of *E. coli* TnaC and SecM contain RF-2 and prolyl-tRNA^{Pro} in the A-site, respectively, and neither paused complex is a substrate for tmRNA [47,48]. Thus, tmRNA-mediated ribosome release appears to be circumvented during programmed translational arrests, allowing these paused ribosomes to regulate gene expression.

Each of the above mechanisms for generating tmRNA substrates involves ribosomes that are stalled at or near the end of an mRNA, and will not produce a correct protein. *trans*-Translation undoubtedly increases the fitness of bacteria by efficiently releasing the stalled ribosomes so they can participate in productive translation, and by targeting the incorrect mRNA and protein for degradation to eliminate them from the cell and provide nutrients. However, deletion of *ssrA* in most species results in specific phenotypes, and not just slower growth. How can these phenotypes be explained? Ideally, the particular activity of tmRNA that is important for each phenotype could be dissected using mutational analysis. It is not possible to completely separate the ribosome release activity from protein tagging and mRNA degradation because tagging requires interaction of tmRNA with the ribosome. However, mutations in tmRNA that alter the last two alanines of the tag peptide, or that truncate the peptide, result in tagged proteins that are significantly more stable than those tagged with the wild-type peptide [20]. Many phenotypes produced by deletion of *ssrA* are complemented by these variant tmRNAs, but some are not [28].

In many cases, phenotypes that are complemented by tmRNA mutants have been attributed to a decrease in translational capacity due to stalled ribosomes stuck on the end of the mRNA. In *E. coli*, the amount of tagging is high enough that, on average, each ribosome translates tmRNA once per cell cycle [26]. Given this frequency of *trans*-translation in the cell, it is certainly reasonable to assume that translational capacity would be severely impaired in the absence of tmRNA if these ribosomes could not be released. In fact, under some *in vitro* conditions ribosomes that translate to the end of an mRNA are extremely stable [19]. However, because *E. coli* cells do not grow significantly slower in the absence of tmRNA, translational capacity cannot be grossly impaired [20]. This conclusion was also readily apparent from studies showing that proteins were efficiently expressed from non-stop messages in *ssrA* cells [26]. Direct evidence that ribosomes are released from non-stop mRNAs in the absence of *trans*-translation has recently been reported. In vitro experiments from the Nierhaus and Inada groups indicate that ribosomes recycle from non-stop messages in the absence of tmRNA. Szaflarski et al. used an optimized cell-free *E. coli* translation system to demonstrate multiple rounds of translation from a homopolymeric poly (U) message [49]. These reactions were conducted under conditions that preclude *trans*-translation, suggesting that ribosomes can recycle from non-stop messages independent of RF and tmRNA activities. Similarly, Kuroha et al. report that 70S ribosomes dissociate from the 3′ end of non-stop mRNA in a completely defined in vitro translation system that lacked tmRNA [50] (Fig. 2). Intriguingly, these dissociated monosomes contain bound peptidyl-tRNAs, which undergo slow spontaneous hydrolysis in the absence of RFs. These results suggest that tmRNA-independent recycling may be an intrinsic property of the ribosome. However, pulse-chase analysis of peptidyl-tRNA turnover shows that paused ribosomes recycle much more rapidly from non-stop mRNA in vivo, perhaps indicating that other factors accelerate recycling [51]. Recycling from non-stop mRNA in *ssrA* cells is not influenced by release factor-3 (RF-3) or ribosome recycling factor (RRF) overexpression, which have been postulated to induce peptidyl-tRNA "drop-off" from the ribosome [52]. Given that the nascent chain is conducted through the 50S subunit exit tunnel, it is difficult to imagine how large, partially folded peptidyl-tRNAs could drop-off of the ribosome. It seems more likely that the nascent chain is hydrolyzed from the P-site tRNA prior to ribosome recycling. Peptidyl-tRNA hydrolase (Pth) has this hydrolytic activity, but is unable to act on ribosome bound peptidyl-tRNA, and recent work has excluded Pth as a player in ribosome recycling [50,51]. Although RF-1 and RF-2 are unable to act on non-stop-arrested ribosomes, *E. coli* has two putative peptide release factors, PrfH and YaeJ, which contain the critical GGQ motif required for peptidyl-tRNA hydrolysis. Each of these proteins lack the stop codon recognition domains of canonical RFs, and therefore could conceivably act in a codon independent manner. Additionally, Ehrenberg and colleagues have demonstrated important roles for IF-1 and IF-3 in alternative recycling, which may be relevant to the tmRNA-independent pathway [53]. Though the details of alternative recycling pathways have yet to be elucidated, these results indicate that ribosomes are not sequestered in the absence of tmRNA. Therefore it is unlikely that the phenotypes associated with mutations in tmRNA are due to decreased translational capacity. However, the tmRNA-mediated release of specific ribosomes, discussed below, may indeed be important.

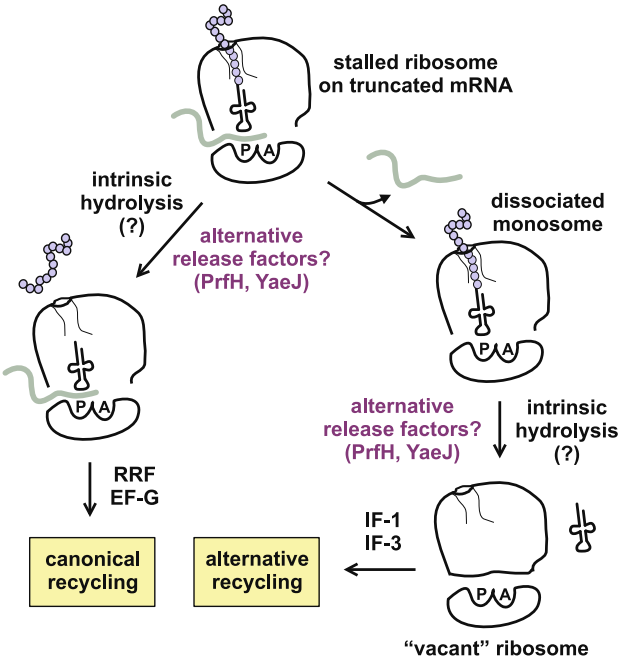

Fig. 2. Postulated tmRNA-independent ribosome release mechanisms. Two pathways are proposed to explain release of ribosomes from non-stop messages in *E. coli* cells lacking tmRNA. The peptidyl-tRNA could be hydrolyzed in the P-site, resulting in a canonical post-termination complex that can be recycled by RRF and EF-G (left). Instead of standard release factors that require a stop codon, this pathway could use an intrinsic peptidyl-tRNA hydrolysis activity recently observed in *E. coli* ribosomes, or hydrolysis may be stimulated by the alternative release factors PrfH and YaeJ, which are proposed to catalyze stopcodon independent nascent peptide release. Ribosomes will also dissociate from the 3′ end of truncated messages in *E. coli* (right). The dissociated monosomes may be processed by the intrinsic peptidyl-tRNA hydrolase activity or alternative release factors to produce a vacant ribosome, which is then recycled by the IF-1/IF-3 pathway.

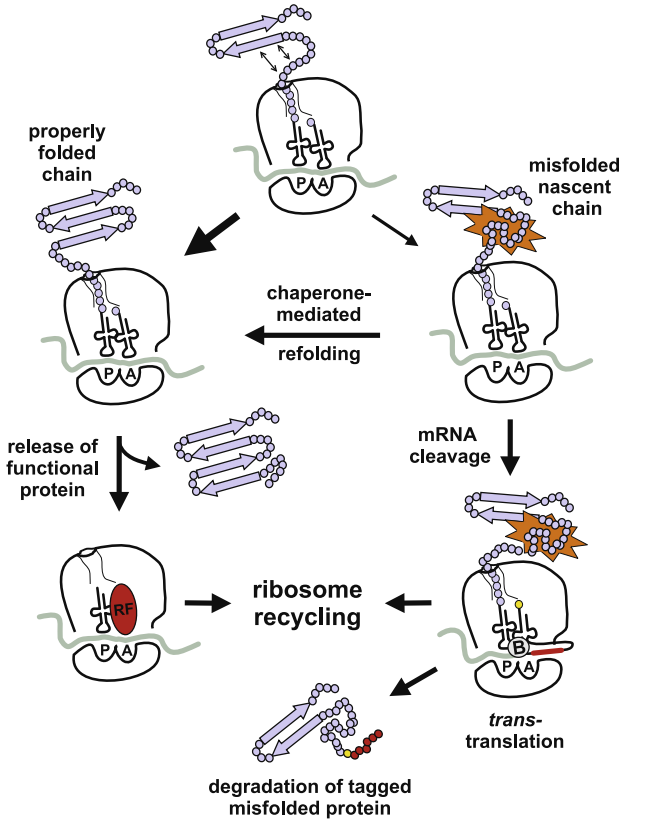

Fig. 3. Co-translational protein folding and tmRNA activity. Translation typically results in a properly folded protein, either through correct folding during synthesis or refolding in conjuction with ribosome-associated chaperones such as DnaJ/K. Disruption of DnaK leads to increased *trans*-translation activity, particularly during the synthesis of large, multidomain proteins. We propose that misfolded nascent chains may induce translational pausing, allowing tmRNA to tag the defective protein for degradation after release from the ribosome.

Despite the progress in understanding mechanisms for targeting translating ribosomes to tmRNA, the physiological significance of many of these mechanisms is unclear. Surveys of proteins that are tagged by tmRNA in vivo have been performed in *E. coli* [45,54], *B. subtilis* [55], and *Caulobacter crescentus* [56], but the mechanisms that produce most tmRNA activity are not known. For example, of the substrates identified in *E. coli*, LacI generates a transcriptional road block [31], RbsK is tagged at rare codons [40], and YbeL, GalE, PhoP, and ribosomal protein S7 are tagged as a result of inefficient translation termination [39,45,54], yet it is not known how many of the hundreds of tagged proteins are targeted for *trans*-translation using these mechanisms. Similarly, of 72 known substrates in *C. crescentus*,only 1 is tagged at a rare codon, and 5 are tagged at the C terminus [56]. It is likely that many tmRNA substrates are tagged for reasons that have not yet been identified. Recent data have suggested that tmRNA substrate selectivity may involve co-translational protein folding (Fig. 3). Several large, multidomain proteins are tagged at tens to hundreds of distinct sites when overexpressed (Z. Ruhe and C.S. Hayes, unpublished results). This tagging is not due to rare codon clusters, mRNA secondary structure, or RNase activity, but is significantly increased by heat shock and deletion of the DnaK chaperone (Z. Ruhe and C.S. Hayes, unpublished results). DnaK can bind to nascent polypeptides to prevent protein misfolding, so one intriguing explanation for the results is that misfolding of the nascent polypeptide promotes ribosome pausing and *trans*-translation. This model is indirectly supported by data showing that thioredoxin, which is soluble and not normally tagged, becomes extensively tagged when fused to a hydrophobic leader sequence that directs it into inclusion bodies (Z. Ruhe and C.S. Hayes, unpublished results). However, DnaK plays several roles in the cell, and it is possible that the increased tagging observed in *dnaK* mutants is not a direct consequence of co-translational misfolding. DnaK is involved in ribosome assembly, and is also associated with the RNA degradosome, which degrades mRNA in *E. coli* [57,58]. Both of these functions have clear implications for tmRNA activity, and therefore the challenge is to unambiguously link protein folding and tagging. A connection between co-translational folding and tagging is especially attractive because it could provide a rationale for some of the other known tagging signals. For example, it is not obvious why inefficient translation termination sequences would be retained through evolution if they decrease the release of otherwise complete proteins into the cell. However, slowing termination may allow time for the nascent protein to fold before it is released from the ribosome, or for the cell to monitor folding and eliminate misfolded protein. If this hypothesis is correct, elongation and termination stalling sequences should be found with high frequency in proteins with complex folding pathways, and these proteins should be tagged by tmRNA more often than proteins that fold efficiently. This prediction remains to be tested. A link between tagging and co-translational folding would also provide an explanation for the constitutive stress response observed in *E. coli* mutants deleted for *ssrA* [59]. If misfolded proteins that are normally tagged by tmRNA and degraded are instead released into the cytoplasm when tmRNA is absent, chaperones and proteases are likely to be up-regulated in response.

If tmRNA activity is linked to co-translational protein folding, is it also involved in other co-translational processes? As noted

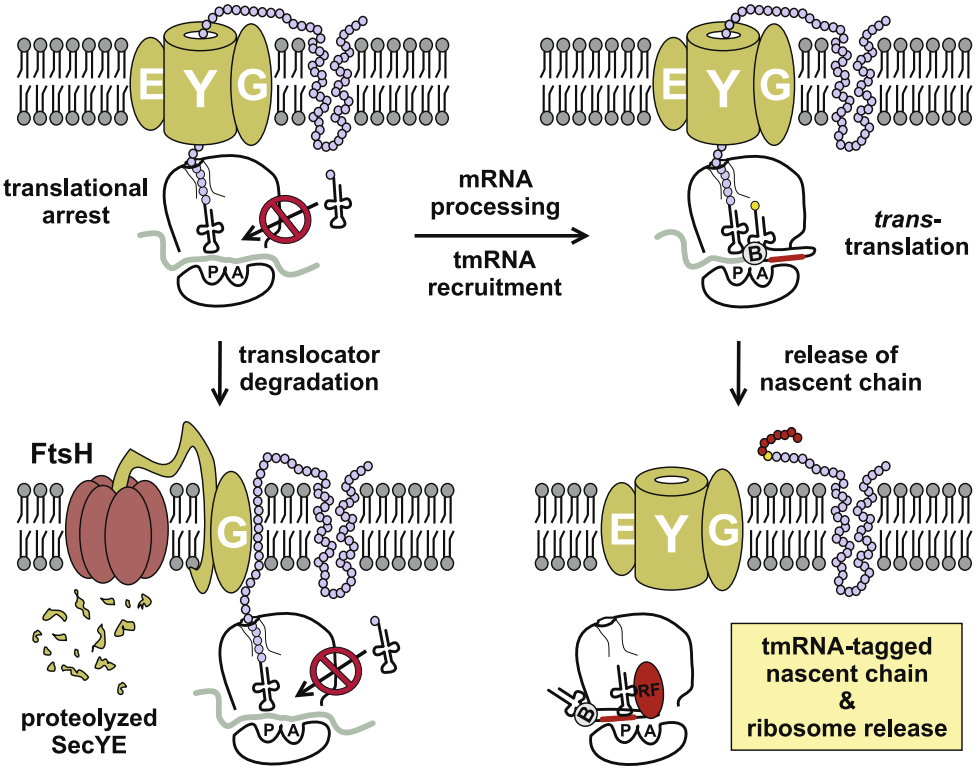

Fig. 4. Hypothetical role for tmRNA in the release of jammed protein translocators. Inhibition of protein synthesis during co-translational protein secretion leads to FtsH-dependent proteolysis of the SecY and SecE components of the translocator [61] (left). tmRNA may help to protect the translocator from degradation by relieving some translational arrests.

above, although current evidence indicates that ribosome release by *trans*-translation is not required to maintain the translational capacity of the cell, release of particularly problematic ribosomes may be important for wild-type physiology. In particular, ribosomes that stall during co-translational protein secretion may cause severe problems for the cell. Most bacterial proteins are secreted through the SecYEG translocator, either during or after translation [60]. When a translocator is blocked, SecY is degraded, and because SecY activity is required to assemble new translocators, an increase in SecY turnover can be lethal or severely impair cell growth [61]. When ribosomes engaged in co-translational translocation are stalled by chloramphenicol or tetracycline treatment, SecY turnover is increased [61]. Presumably the translocator can pull on the nascent polypeptide, but the ribosome is much too large to be secreted. It is not known what happens during co-translational translocation if the ribosome stalls on a non-stop mRNA, but an attractive model is that *trans*-translation activity allows a tagged protein to be translocated, saving SecY from destruction (Fig. 4). Alternative ribosome release mechanisms that result in dissociation of the peptidyl-tRNA from the ribosome in the absence of tmRNA might not prevent SecY turnover, because the peptidyl-tRNA is also likely to block the translocator. This translocator-jamming model would explain why tmRNA mutants that do not promote rapid proteolysis of tagged proteins would frequently complement the deletion phenotype: any tag would free the blocked translocon, as long as the nascent polypeptide is released. The subcellular localization of tmRNA and SmpB is also consistent with a link between *trans*-translation and protein secretion. Even though ribosomes are found throughout the cytoplasm, tmRNA and SmpB are concentrated in a helix-like structure similar to that observed for SecY, SecE, and SecG [62–64]. Because all known bacteria use the SecY translocator, a role for tmRNA in freeing blocked translocators could provide an explanation for its universal conservation. Another universal role for *trans*-translation in releasing specific stalled ribosomes has been proposed by Pomerantz and O'Donnell [65]. When DNA polymerase collides with an RNA polymerase transcribing in the same direction, the replication fork collapses. After removal of RNA polymerase, replication restarts using the mRNA transcript as a primer. Thus, stability of the mRNA and maintenance of the RNA-DNA hybrid are critical to efficient restart of replication. Translation of the mRNA would be likely to interfere with replication restart, so it would be beneficial to free these ribosomes from the mRNA when RNA polymerase is removed. Although the in vitro studies described above suggest that tmRNA will not enter the ribosome when there is mRNA extending past the leading edge, it is conceivable that interactions between the ribosome and RNA polymerase could facilitate entry of tmRNA in these complexes after collision. The release of other stalled co-transcriptional translation complexes, such as those produced during a head-on collision with DNA polymerase or after transcriptional arrest, might also involve *trans*-translation. Clearly, many functions of tmRNA remain to be unraveled.

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